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A STUDY OF THE HEMOLYTIC ANTIBODY-ANTIGEN COMBINATION

DISSERTATION

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in Conformity with the Requirements for the Degree

of Doctor of Science in Hygiene.

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A STUDY OF THE HEMOLYTIC ANTIBODY-ANTIGEN COMBINATION.

I. Introduction.

According to Ehrlich (1), a formed antigen, such as a red cell or bacterium, has a definite number of combining groups which have an affinity for the antibody. When the antigen is placed in contact with its antibody, a reaction ensues, which progresses until all these bonds are satisfied or until all the antibodies have entered into the combination. It follows from this that when all the combining affinities of the antigen are satisfied, no more antibodies can enter into the reaction regardless of the number that still remain free in the supernatant fluid. This theory implies chemical affinities and a combination according to the respective valencies of the reacting substances.

The view of Arrhenius was opposed to this. He regarded the reaction as physical (2), and explained the phenomenon as a distribution of a solute between two solvents, the antibodies being the solute, and the cell protoplasm and the surrounding fluid the two solvents. He concluded that "the immune bodies are probably not bound by the erythrocytes but only absorbed by them", and that "no proof has been given of their chemical action."

Bordet's idea (3), although not the same as that of Arrhenius, was similar in that he attempted to explain the phenomenon according to physical laws. He, however, held that the antibody or "sensitizing substance" is adsorbed in much the same way that a filter paper takes up a dye. He states that "the union of the antibody with the antigen depends on what is called molecular adhesion or contact affinity, in other words, should be classed in the category of adsorption phenomena."

It has long been known that a red cell or bacterium will combine with much more than the amount of antibody necessary for lysis. Arrhenius found that the amount of antibody taken up increases with the concentration according to a definite physical law, which he expressed by the equation,

$$B = K C^n,$$

in which B represents the amount of antibody absorbed by the cells, C the concentration remaining in the supernatant liquid after absorption, and K and n are constants. He found the value of n to be 2/3, and from the equation and the calculated value of n, he interpreted the reaction as a distribution of the antibodies

between the red cell protoplasm and the surrounding liquid as solvents, and that two of the antibody molecules free in the serum form three of the combined molecules.

Manwaring's work (4-8) on this same phenomenon failed to confirm the conclusions of Arrhenius. He found that the absorption did not follow any simple physico-chemical law, and that K and n were not constants. Manwaring obtained what he calls a "negative absorption", i.e., the titer of the serum dilutions, when great concentrations of antibody units were used, was often greater after contact with the corpuscles than before. He concluded, therefore, that qualitative changes take place in the amboceptor due to its contact with the cells,and "that any direct quantitative comparison between it and the untreated serum gives erroneous results."

From these and other experiments, he came to the conclusion that there is a "third component" in the serum besides the antibody and complement, which varies in quantity in different animals. This third component may be "antilytic", or "auxilytic", but never has independent hemolytic powers, although it may be absorbed by the red cells. In addition, he mentioned several other factors influencing the antigen-antibody combi-

nation, among which are the reaction and specific gravity of the medium and the amount of inorganic salts.

Amato (23), in a recent study of opsonic sensitization of bacteria, has come to the conclusion that the union of the opsonins with the bacteria is governed by the same law that governs the union of hemolysins with the antigen. He found the same equation applicable to the opsonins which Arrhenius applied to the hemolysins and concluded that the reaction is probably a distribution of the opsonins between two solvents in which they have different molecular weights.

Coulter (9 and 10) has done some very definite work on the influence of the reaction of the medium on the absorption of hemolytic sensitizer by red cells, and on the dissociation of the combination. He found pH = 5.3 to be the optimum H-ion concentration for the absorption. However, he also found very little difference in absorption between the values, pH = 4.5 and pH = 6.0, in the salt-free medium, and a much wider range in medium containing salt.

Kahn (11), in a very recent work on the absorption of hemolytic sensitizer, has made some studies on the rate of the reaction at the various temperatures. He finds that the reaction is completed very quickly, in every case at the end of fifteen minutes. He also finds

"that the extraction is greater at 37° than at room temperature, which in turn is greater than at ice-box temperature."

It is seen then that the antigen-antibody combination is influenced by time, temperature, the reaction of the medium, and the amount of inorganic salts. In addition, there is also considerable variation due to uncontrolled factors, which vary with the different lots of serum used -- those variations which caused the differences of opinion between Arrhenius and Manwaring.

In the face of all that has been done on the absorption of hemolytic antibodies by red cells, it would seem almost hopeless to try to add anything new, either in fact or theory. However, it is thought that the results of the experiments recorded in this paper are significant in suggesting an explanation of the differences obtained by Arrhenius and Manwaring, without the necessity of resorting to the sub-divided "third component", or to any qualitative changes in the amboceptor due to its contact with the red cells.

In all the discussions the terms, "amboceptor", "antibody", "sensitizer", and "immune body", will be used interchangeably.

Experimental.

II. The Concentration of the Hemolytic Antibodies as a Factor Influencing their Absorption by Red Cells.

These experiments were carried out in order to determine how many times the amount of sensitizer necessary for hemolysis the red cell will absorb, and whether or not there is a definite saturation point, as might be assumed from Ehrlich's theory, above which the red cells will absorb no more, regardless of the amount still remaining free in the serum.

The technic was as follows: Rabbits and guinea-pigs were immunized to the red cells of the sheep, by intraperitoneal injections of the guinea-pigs and intravenous injections of the rabbits. The serum so obtained was inactivated at 56°C. for 30 minutes and then carefully titrated against fresh sheep cells that had been collected in one per cent. sodium citrate and washed four times in large volumes of normal salt solution. The unit of cells was arbitrarily chosen as 0.1 cc. of a 1/4 * suspension (measured in terms of whole blood), and the unit of complement as 0.1 cc. of a 1/5 dilution of fresh

* All dilutions and suspensions were made in normal salt solution. A 1/4 suspension of cells means one part of cells plus three parts of salt solution.

normal guinea-pig serum, pooled from several animals. The unit of antibody or sensitizer was defined as the smallest amount of the immune serum which, under the above conditions, would just suffice to hemolyze a unit of sheep corpuscles in a total volume of 1 cc., in one hour's incubation at 37°C. Protocol 1 will illustrate.

Protocol 1. Type for Hemolytic Titrations.

Tubes	1	2	3	4	5	6	7	8	9
Immune	* 1/200				1/500				
serum	0.5	0.4	0.3	0.25	0.5	0.4	0.3	0	0
Guinea-pig	1/5								
complement	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0
Sheep cells									
1/4	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Salt									
solution	0.3	0.4	0.5	0.55	0.3	0.4	0.5	0.8	0.9
Hemolysis									
1 hour **	4+	4+	4+	4+	4+	3+	2+	0	0

* The numbers, 1/200 and 1/500, represent the dilutions of the immune serum used in the tubes. For instance, tubes 1 to 4 received 0.5, 0.4, 0.3, and 0.25 cc. of a 1/200 dilution, respectively.

** 4+ means complete hemolysis. The degrees of hemolysis are indicated by the signs, 3+, 2+, and +.

In the above sample titration, it is seen that the hemolytic unit of this particular serum is 0.5 cc. of a 1/500 dilution, or 1/1,000 cc. Then 1 cc. of this serum contains 1,000 hemolytic units. The titer is expressed as 1/1,000, or sometimes as 1,000.

In making the absorption tests, a number of tubes

Protocol 2. Type for Absorption of Hemolytic Units by Sheep Red Cells. *

Tube number	Sensibizer Units per cc.	Sensibizer Quantity of serum	Sheep cells 1/4	Salt sol.	Titer after absorption	Units absorbed
1	5	1 cc. 1/400	0.2	0.8	0	5
2	10	0.2 cc. 1/40	0.2	0.6	0	10
3	20	0.4 cc. 1/40	0.2	1.4	0	20
4	50	1.0 cc. 1/40	0.2	0.8	1/2	48
5	70	1.4 cc. 1/40	0.2	0.4	1/8	62
6	100	0.2 cc. 1/4	0.2	1.6	1/12	88
7	200	0.4 cc. 1/4	0.2	1.4	1/40	160
8	500	1.0 cc. 1/4	0.2	0.8	1/150	350
9	1000	0.5 cc. undil.	0.2	1.3	1/400	600
10	2000	1.0 cc. undil.	0.2	0.8	1/1000	1000
11 **	50	1.0 cc. 1/40	0	1.0	1/50	0
12	500	1.0 cc. 1/4	0	1.0	1/500	0

* All the sets were made in duplicate.

** Sets 11 and 12 are controls.

were arranged, each containing two units of sheep cells, and sensitizer in varying amounts. Enough salt solution was added to each tube, before the sensitizer, to make the final volume two cubic centimeters. The tubes were incubated in a water bath for 30 minutes at 37° C., after which the red cells were removed by centrifugalization and the supernatant fluid titrated as in Protocol 1, to determine the number of units of sensitizer lost, i.e., the number removed by the red cells. The use of all the materials concerned in the reaction in only twice the amount ordinarily used for titration overcame the necessity for the use of an undue amount of immune serum and still afforded sufficient supernatant fluid for the subsequent titration. The method is shown in Protocol 2.

Tables I and II show the absorption from immune rabbit and guinea-pig serum in concentrations ranging from 5 to 2,000 units per cubic centimeter. On the first line of each table are shown the concentrations with which the cells were treated, and on the following lines are shown the amounts of antibody taken up by the cells from each concentration in the tests made with the different lots of serum. Some of the results are also shown graphically in Figs. 1, 2, and 3, in which are

Table I. Absorption of Antibodies from Rabbit Immune Serum by Sheep Erythrocytes.

Concentration of antibody units per cc. in the serum dilutions.		20	50	100	200	500	1000	2000	Remarks.
Units absorbed.	Test # 1	20	49	90	180				
	" # 2	20	48	92	175	340			Titer 1/10000.
	" # 3	20	47	86	160	300	360		Serum of test 2, stored 4 months, titer 1/6000.
	" # 4		47	90	167	375	500	670	
	" # 5		47	92	150	375	500		
	" # 6	19	47	88	150	300	500	750	
	" # 7	18	30	44	77				Titer 1/1000.
	" # 8	20	49	97	180	300			
	" # 9		49	95	193	450	700	1200	Titer 1/40000.
	" #10		49	98	185	350	540	875	Serum from same rabbit as in test 9, 8 days later. Titer, 1/25000.
	" #11		47	87	150				

Table II. Absorption of Hemolytic Antibodies from * Immune Guinea-pig Serum by Sheep Erythrocytes.

Concentration of antibody units per cc. in the serum dilutions.		5	10	20	50	100	200	500	Titer.
Units Absorbed	Test # 1	5	10	19	41	70	100		1/4000
	" # 2	4	7	12	20	40	70		1/2500
		Serum of test 1, stored 4 months.							
	" # 3	4	6	12	25	40	70	235	1/1000
	" # 4	4	6	10	20	33	40	100	1/1000
	" # 5	4	5	8		50			1/1000
	" # 6	3		4	10	20			1/125
	" # 7				17	20	33		1/2000

* From 1000 units sensitizer from guinea-pig serum the absorption was so often difficult to determine that it is not listed here. Often no difference in titer could be detected after contact with the cells.

plotted the logarithms of the amounts absorbed against the logarithms of the concentrations remaining in the liquid after the absorption is completed. The plot, as is seen, tends to approach a straight line.

A study of Tables I and II brings out several important facts. Even from comparatively low concentrations of antibody units all are not absorbed, while, if the concentration in the liquid is sufficiently high, massive quantities are taken up. The only instances in which the reaction seems to be complete are when very low concentrations are used, and even here, the assumption that all the sensitizer is absorbed does not seem justified, because amounts much less than one unit per cubic centimeter could not be detected by the method of titration used in these experiments. It is evident, therefore, that the number of amboceptor units taken up by the sheep red cells varies with the concentration of those units in the surrounding medium; the more concentrated the units are, the more are absorbed.

Another fact to be noted is that the absorption is nearly always higher from the rabbit serum than from the guinea-pig serum, and that the absorption varies with the serum of individuals of the same species. This will be referred to later.

Explanation of the Figures:

Fig. 1. Logarithmic plot of the data of Table III. The amount of sensitizer absorbed is plotted against the concentration remaining in the supernatant liquid. Titer of serum, 1/4,000. K = 22.18, n = 0.5.

Fig. 2. Curves A and B, logarithmic plots of the data of tests 3 and 4 of Table II. The data for these plots are also shown in Table VI. For Curve A, K = 3.225, n = 0.78. * For Curve B, K =1.97, n = 0.61.

Fig. 3. Logarithmic plots, A and B, representing the data of Tables IV and V. The sera were obtained from rabbit 69 on the 10th and 24th days respectively. For Curve A, K = 165.9, n = 0.36. For Curve B, K = 80.73, n = 0.4.

Fig. 4. Logarithmic plot of the data of test 5, Table IX. K = 65.32, n = 0.37.

Fig. 5. Similar plot of test 12, Table IX. K = 35.0, n = 0.51.

* Where the plot deviates from the straight line the values of K and n can be determined only approximately.

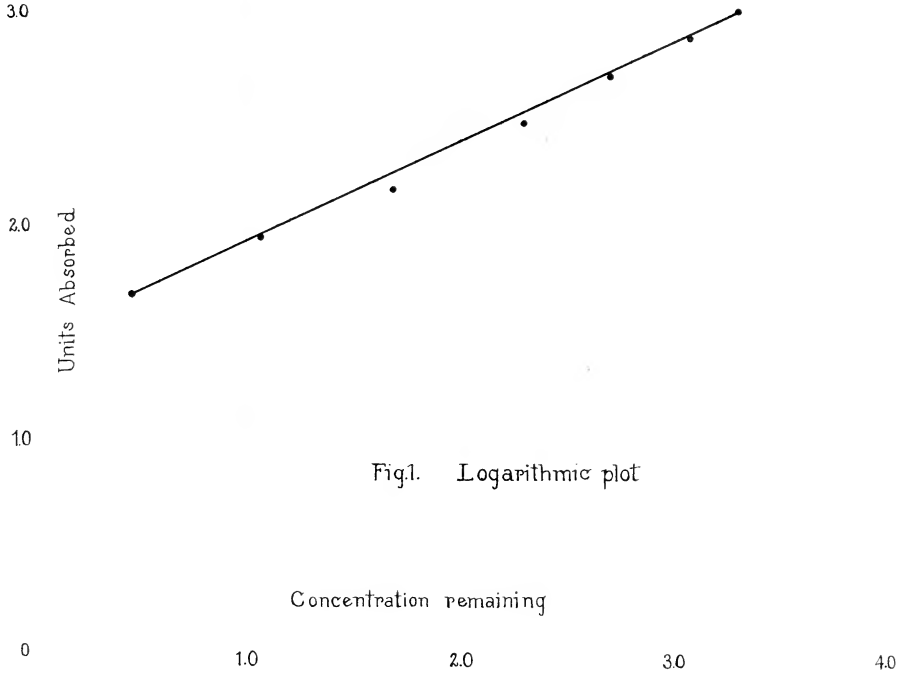


Fig.1. Logarithmic plot

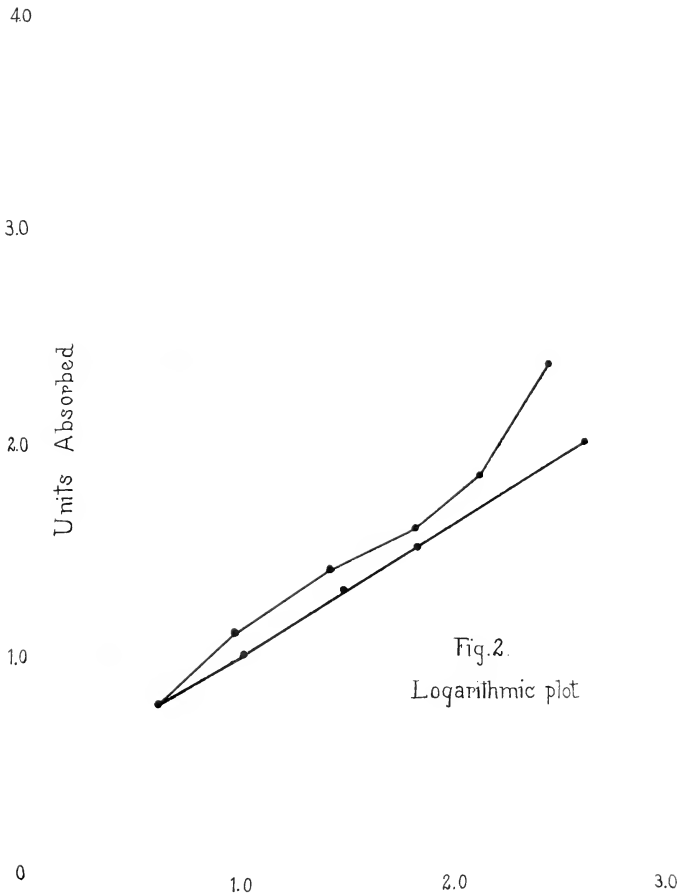


Fig.2.
Logarithmic plot

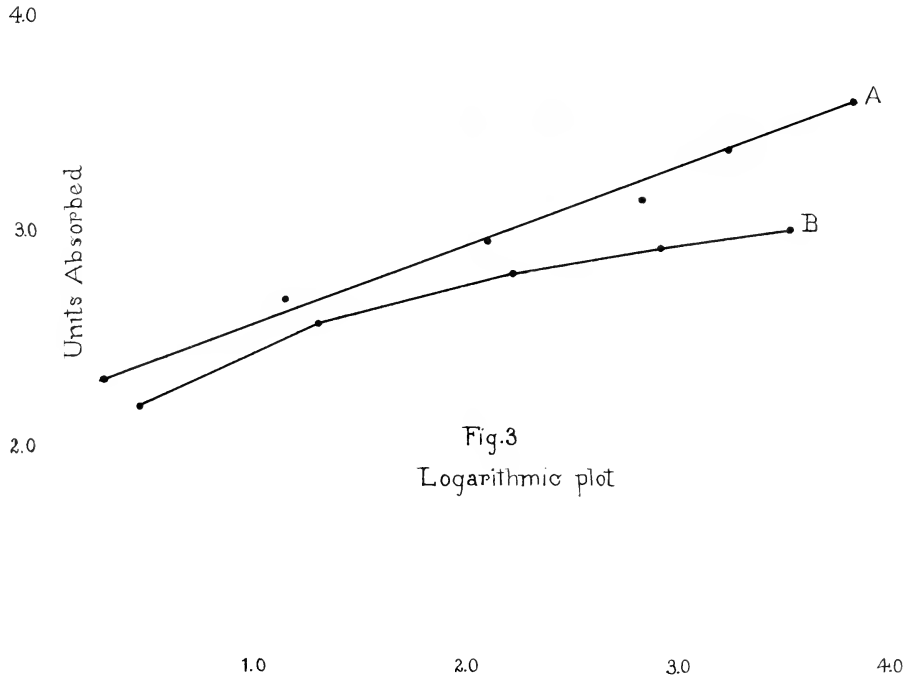


Fig.3
Logarithmic plot

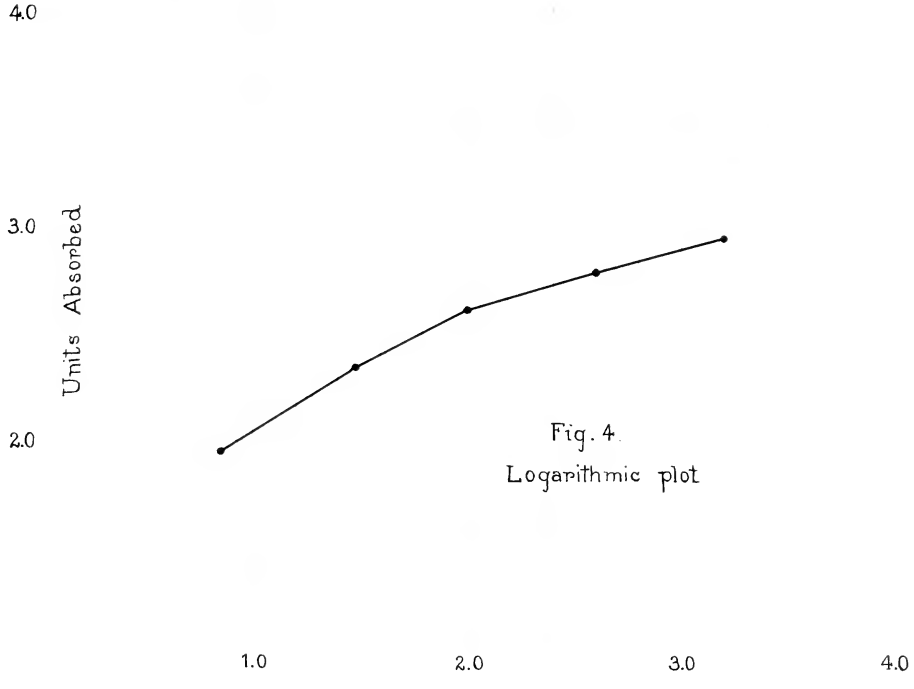


Fig. 4
Logarithmic plot

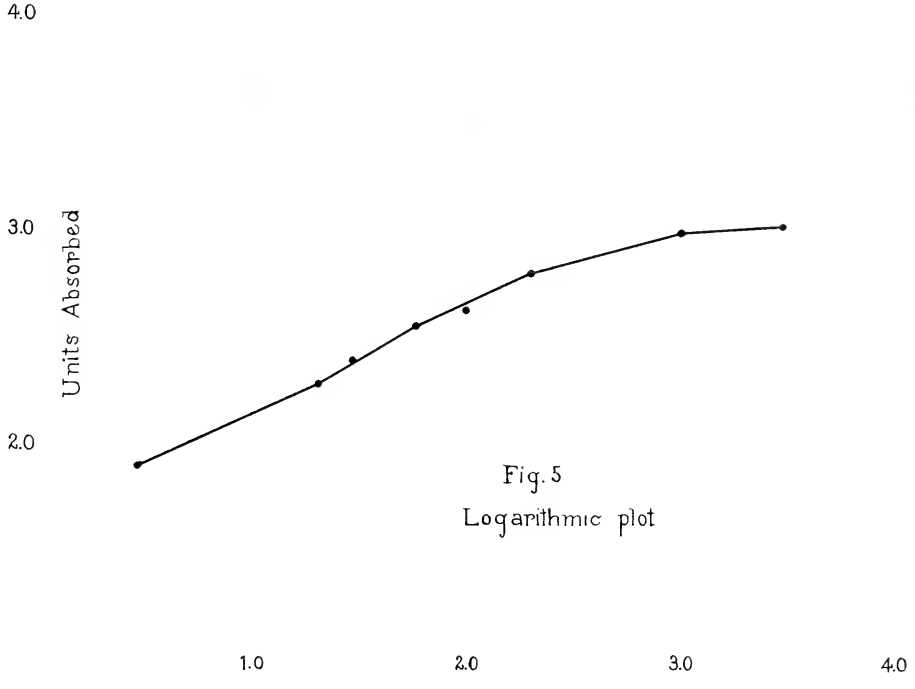


Fig. 5
Logarithmic plot

The results here recorded agree, in part, with those obtained by Arrhenius and Morganroth (2) in their work on the absorption of immune bodies by ox and sheep cells, in which they were able to express the reaction according to the equation,

$$B = K C^{n}.$$

This equation holds true, however, only when the logarithmic plot, as shown in the figures, represents a straight line; and the farther it deviates from the straight line, the more inapplicable becomes the equation, because of the variation in the values of the constants, K and n, between the different concentrations. The tests represented by Figs. 1 and 3A show practically straight line plots, so here the equation can be applied. The experimental and calculated results compare very favorably, as is shown in Tables III and IV, which correspond to Figs. 1 and 3A, respectively. The disagreement is, in most cases, within the limits of experimental error. The values of the constants, K and n, should be noted. The "absorption constant", K, is 22.18 for Table III, and for Table IV, K = 169.5, which is very much higher. The absorption is also very much higher from all concentrations in Table IV. The value of n differs for the two tables, and in both it is very much

Table III. Experimental and Calculated Results from Test 6, of Table I.

B *	C (obs.)	C (calc.)	K	n
46	4	4.4	22.18	0.5
88	12	15.7		
150	50	45.7		
300	200	170.3		
500	500	508.2		
750	1250	1144.0		
1000	2000	2034.0		

Table IV. Experimental and Calculated Results from Absorption Tests Made on the Serum of Rabbit 69, Obtained on the 10th Day.

B	C (obs.)	C (calc.)	K	n
198	2	2	165.9	0.36
487	13	24		
875	125	124		
1340	660	408		
2350	1650	1950		
4000	6000	8000		

* B = the number of units absorbed by the cells,
C = the concentration remaining after absorption.

Table V. Results of Absorption Tests on the Serum of Rabbit 69, Obtained on the 24th Day.

B	C (obs.)	C (calc.)	K	n
157	3	5	80.73	0.4
380	20	15		
630	170	170		
800	800	309		
1000	3000	540		

Table VI. Experimental and Calculated Results from Absorption of Sensitizer from Guinea-pig Immune Serum by Sheep Erythrocytes.

Curve A, Fig. 2.

B	C (obs.)	C (calc.)	K	n
6	4	2.54	3.225	0.78
12	8	7.19		
25	25	21.52		
40	60	43.68		
70	130	101.20		
235	165	622.10		

Curve B, Fig. 2.

6	4	5.3	1.969	0.61
10	10	11.5		
20	30	32.8		
33	67	68.9		
40	160	91.6		
100	400	362.3		

lower than the value, 2/3, which was found by Arrhenius. In Fig 1, n = 0.5, and in Fig. 3A, n = 0.36, i.e., the the curve having the steeper gradient has also the higher value for n. Thus, it is seen that the constants vary with the different lots of serum used, the value of K, determining the position of the curve, and of n, the gradient.

In Fig. 2, there are shown two plots, A and B, representing the two tests shown in Table VI. Curve A deviates considerably from the straight line so the values, K = 3.225, and n = 0.78, are necessarily calculated only approximately from the averages of several determinations made at intervals along the curve. There is also in the table quite a discrepancy between the observed and calculated results. The table for curve B, which approximates the straight line very closely, shows a very close agreement between the observed and the calculated results.

III. The Rate of the Reaction between the Antigen and Antibody, in vitro.

It would seem that the union of the antigen and antibody is extremely rapid, and practically instantaneous in vivo. This is demonstrated by the anaphalactic shock in hypersensitive animals, for when the antigen is injected into the blood-stream, the shock often occurs immediately. Bull (12) has found pneumocccci, when injected into the blood-stream of an immune animal, to be agglutinated within a very few seconds.

In the previous absorption experiments, the cells were allowed to remain in contact with the sensitizing serum for 30 minutes, which was considered sufficient time for the reaction to reach equilibrium. In order to control this, however, it was necessary to establish definitely whether or not the time allowed was enough, and how much it could be varied without influencing the results.

In this experiment, sheep cells and sensitizer were allowed to remain in contact for varying periods of time and the amount taken up tested by titration, as in the previous experiments. The longest time was two hours. The shortest time, which is listed as one minute in the

Table VII. Rate of the Antigen-Antibody Reaction.

Concentration of antibody units per cc. in the serum dilutions.	100	100	165	250	500	2000	Time.
		60	65	80	250		1 min.
	84	82	100	120	350	400	5 "
	87	88	100	125	375	400	15 "
Units Absorbed.	87	88	100	125	375	400	30 "
	90	90	100	125	375	400	60 "
	87	88	100	125	375	400	120"

table, could not be kept absolutely constant. The corpuscles were added to the serum dilutions, shaken up thoroughly, and immediately centrifuged at high speed. The time during which the cells were in free contact with the serum was certainly not more than two minutes in any case. The results are given in Table VII.

It is evident, from this table, that equilibrium is reached very quickly. It is practically complete within five minutes, and entirely so in every case at the end of fifteen minutes. A slight dissociation, at the end of two hours, is indicated in the absorption from 100 units, but this is not very pronounced and does not show at all in the higher concentrations. The data given here are few, but the experiment serves its purpose as a control to the other experiments, and shows that the time allowed for the absorption may be varied within a wide range without materially influencing the results.

IV. The Influence of Temperature on Absorption of Antibodies by Red Cells.

A temperature of 37°C. is employed in most serological reactions. The combination of the antigen and antibody, however, takes place at much higher and much lower temperatures. Differences of opinion occur among the different investigators, as to the best temperature for the combination. Neil (13) and Kolmer (15) advise the use of room temperature as the best for the sensitization of cells, while Hinton (14) recommends 37°C. These opinions were advanced as the result of rather extensive work on the standardization of the Wassermann Reaction. Kahn (11) states that "the extraction is greater at 37° than at room temperature, which in turn is greater than at ice-box temperature."

In the experiments here recorded, the influence of the variation of the temperature on the absorption of hemolytic antibodies has been observed. The absorption tests were carried out as in the foregoing experiments, using the rabbit anti-sheep hemolytic system. Duplicate tests were made for each temperature and concentration. The antibody dilutions were made first, and the cells added after both had been brought to the required temperature. It is needless to

Table VIII. The Influence of Temperature upon the Antigen-Antibody Reaction.

(A).

Serum	A	B	C			D	
Concentration of antibody units per cc. in the serum dilutions.	100	600	65	130	320	400	Temperature.
	92	325	55	105	120	108	0°C.
Units	95	475	60	120	190	175	15 "
	96	475	62	120	200	180	25 "
Absorbed	97	475	62	130	220	220	37 "
		500					40 "
		475					45 "
		440					50 "
		400					55 "

(B). Serum of Rabbit 487.

Concentration of antibody units per cc. in the serum dilutions.	100	100	200	200	500	800	Temperature.
	92	93	180	178	330	580	0°C.
	95	95	187	183	340	600	15 "
Units	95	95	187	184	340	600	25 "
	90	93	180	178	330	575	37 "
Absorbed	88		175	167		575	40 "
	83		163	160	320	550	45 "
	75		155	150	310	450	50 "
			120		300	400	55 "
			75			300	60 "

Table VIII. Continued.

(C). Absorption from 100 Units of Sensitizer from the Sera, E to I.

Temperature		0°	Room	37°	45°
Absorption	E	88	94	95	90
	F	93	96	94	97
from	G	100	100	100	99
	H	40	50	55	45
Sera	I	50	70	60	50

(D). Absorption from 200 Units of Sensitizer from the Sera, E to I.

Temperature		0°	Room	37°	45°
Absorption	E	145	170	185	175
	F	167	183	170	155
from	G	199	200	200	199
	H	120	160	165	135
Sera	I	80	120	100	100

say that the tests at all different temperatures with one lot of serum were made on the same day with the same lot of cells and complement. The results are given in Table VIII.

In the tests with the sera A, B, C, and D. the absorption was greater at 37° than at the lower temperatures. That is, the absorption at 37° > 25° = or > 15° > 0°. In the tests with the serum of rabbit B, the absorption was greater at 40° than at any other temperature.

The serum from rabbit 487, however, when tested gave a different result. Seven tests were made on this one lot of serum at different times, using the various concentrations listed in the table. In every test, the temperature of 15° to 25° proved to be the optimum, lower absorptions being obtained at 0°, and 37°, and above. The relative amounts of absorption for this serum may be expressed thus: 15° = 25° > 0° = or > 37°. It is true that the differences in absorption between 15° and 37° are not great, but they consistently appear in all the concentrations tested.

The results obtained with this serum suggested that the optimum temperature for the absorption might be a variable, which is different for the different sera. Therefore, other lots of serum, which had been kept stored with phenol, were tested to see if any would fall

into the same class with that of rabbit 487. These are listed in the table as sera E to I, inclusive. The last two, H and I, were from guinea-pigs and the rest were from rabbits. Table VIII, (C) shows the absorption from 100 units of sensitizer from each of these sera at the different temperatures. In Table VIII, (D), is shown the absorption from 200 units. Of these four sera, F and I proved to be in the same class as that of rabbit 487, i.e., about room temperature is the optimum for absorption.

Those investigators, therefore, who claim that either room temperature or 37° is the best for the sensitization of the cells, were doubtless right in their observations, but any generalization that all sera react in the same way is obviously incorrect. The best temperature for the sensitization of the cells seems, rather, to be a variable, its absolute value depending on the particular lot of serum used in the test. No explanation of this variability is here advanced.

V. The Relation of the Globulin Content of the Immune Serum to the Absorption of the Antibodies.

It was noted in Section I that the absorption of hemolytic antibodies by sheep cells varies with the sera of different animals of the same species and of different species. The question at once arises as to the cause of this variation.

Manwaring (16) has shown that serum proteins may be absorbed by the red cells of another species. It has long been known that the immune bodies are, in most cases, thrown out of the serum along with the globulin fractions, and Hurwitz and Meyer (17 and 18) have shown that these globulins are, as a rule, although not necessarily, increased in varying amounts during the process of immunization. The increase is, in their opinion, due to, and roughly proportional to the amount of metabolic disturbances set up in the animal. The evident relation of the globulins to the antibodies, in these respects, suggested a possible connection with the absorption of the antibodies by the cell-antigen. The experiments recorded in this section were undertaken with this idea in mind.

The sera of rabbits and guinea-pigs were used in this work, and quantitative determinations of the

Table IX. Absorption from Immune Sera with Quantitative Determinations of the Serum Proteins.

Concentration of antibody units per cc. in the serum dilutions.		20	50	100	200	500	Titer	Per cent. Glob.	Per cent. Alb.
Test	Units absorbed in Animal Rabbit								
1	276		50	99	193		8000	1.71	5.73
2	374		45	80	150		2000	1.82	4.26
3	471		47	87	150		2500	1.16	5.6
4	487		49	95	193		40000	1.48	5.62
5	481			97	175		10000	1.95	4.48
6	481		30	50	91	160	1500	3.24	2.63
7	481	18	30	55	100		800	2.7	4.18
8	481	15	23	37			600	2.74	4.76
9	481	18	30	55	102		500	2.34	4.6
10	481	17	35	65	120		500	2.81	4.12
11	481	20	42	76	140		500	3.92	4.15
			Animal emaciated, died soon after.						
12	486			94	180	410	8000	2.41	4.01
13	486		30	52	91	160	1500	3.47	2.73
14	486		40	80	150		1000	2.2	4.5
15	486	19	38	71			800	1.96	5.38
16	486	18	30	55	102		500	2.19	4.8
17	486	17	35	65	120		600	2.77	3.85
18	486	18	40	71	134	370	600	3.42	4.25
			Animal emaciated, died soon after.						
19	Guinea-pig.	15	25	40	45		2000	2.51	2.48
20	"	15	20	30	40		1500	2.65	2.08
21	"		17	20	33		2000	3.03	2.08

serum proteins were made on each lot of serum before the absorption tests were done. The protein determinations were made according to the microrefractometric method of Robertson (19) , with an Abbe refractometer. Serum, inactivated at 56° C., was always used fresh to avoid the possibility of bacterial contamination, and special attention was given to the cleaning of the glass-ware.

The results of this work are given in Table IX. * There is no evidence, from these, that the amounts of the serum proteins present have any relation to the amount of sensitizer absorbed by the cells. Rabbits 481 and 486 were bled and tested at intervals for 116 days after the beginning of the immunization. The globulins and albumins fluctuated considerably during that time, but the fluctuations seem not to bear any evident relation to the absorption. At the time of the last bleeding both animals were emaciated and the globulins of both sera were very high. They were about the same in amount as at the second bleeding, but the absorption is much more complete.

* The figures given in Tables IX to XII do not all represent the actual experimental results. In many cases the concentrations tested did not coincide with those given in the tables, so the figures had to be interpolated from the logarithmic charts, for the purpose of tabulation.

VI. The Relation between the Absorption of the Antibodies and the Titer of the Serum.

It has been noted that the constants, K and n, vary in the absorption from the sera of different animals. It has also been observed, incidentally, that often the serum of the higher titer showed the higher absorption. In tests 2 and 3, of Table I, the serum was tested fresh and then tested again after four months' storage, during which time the titer dropped from 10,000 units to 6,000 units per cc. The later test gave a somewhat lower absorption than when the serum was first drawn. Another serum was tested with a titer of 40,000 units per cc., and gave a very high absorption (test 9, Table I). The rabbit was kept and its serum drawn again 8 days later (test 10, Table I). The titer had dropped to 25,000 units per cc., and from all concentrations a marked lowering of the absorption was observed. It was thought, therefore, that there might be a relation between the titer of the serum and the number of antibody units the red cells would take up.

A number of fresh sera of both high and low titers were tested. Some of the animals having serum of high titer were tested subsequently, after the titer had dropped. The results are given in Table X. In

Table X. Relation of the Absorption to the Titer of the Serum.

Concentration of antibody units per cc. in the serum dilutions.		10	20	50	100	200	500	Titer
Test	Units absorbed in							
1	Guinea-pig		4	10	20			125
2	"	5	8		50			1000
3	"	6	10	20	33			1000
4	"	6	12	24	40			1000
5	"	5	19	41	70	100		4000
6	Rabbit	10	20	50				50
					Normal, untreated.			
7	"	10	18	30	44	77		1000
8	"	10	20	50	100			100
					Normal, untreated.			
9	"			45	80	150		2000
10	"		18	35	55	100		800
11	"	10	19	37	70	125		800
12	"	10	20	47	92	160	340	10000
13	"			47	90	167	375	6000
14	"	10	20	47	86	160	300	6000
15	"			47	92	150	375	4000
16	"		19	46	88	150	300	4000
17	"72			50	100			100
					animal three days immune.			
18	"72				99	194	450	1000
					animal six days immune.			
19	"72					198	485	16000
					animal ten days immune.			
20	"80				91	175	350	800
					animal five days immune.			
21	"80				96	188	426	8000
					animal nine days immune.			

many cases, the absorption seems to vary with the titer of the serum, but this is not constant, and the differences in titers above 2,000 units per cc. seem to have no definite relation to the absorption. Normal rabbit serum, in spite of its low titer, gives complete absorption from the undiluted serum.(Tests 6 and 8). Most of the other low-titered sera were obtained from rabbits that had formerly had high titers, and the absorption from these was uniformly low. Tests 17 to 21, however, were made with the sera of rabbits 72 and 80, drawn during the first few days of immunization. They all show uniformly high absorption, although the titers vary from 100 to 16,000 units per cc.

These results seem to indicate, therefore, that, although in many cases, the higher the titer the higher the absorption, it is not the titer of the serum, per se, which causes the variation. Rather, it would indicate that the extent of absorption is dependent on the time the animal is immune, the higher absorption being obtained early in the period of immunity. This variation may be caused by some inhibiting substance which is present in the serum in larger quantities later in the immunity period.

VII. Selective Absorption.

In the preceding section, it was indicated that the variation in absorption may be due to some disturbing element in the serum, the lowering of the absorption depending on the amount of this substance present. It was not possible to show any influence of the fluctuations of the serum proteins, so evidently the disturbance is not due to the serum proteins as such. The problem now is to determine the nature of this element, if possible, and its mode of action, i.e., whether it is taken up by the cell, or acts merely by virtue of its presence in the serum.

It has been noted before that, when the logarithmic plots of the absorption are made, some of the curves represent straight lines while others do not. Curve A, Fig. 3, for which K = 165.9, approaches the straight line very closely. Curve B, in the same figure, represents a test made on the serum of the same rabbit, drawn 6 days later. The titer had dropped from 20,000 units to 16,000 units per cc. with a drop in the absorption constant, K = 80.73. The plot, as is seen, deviates considerably from the straight line, the deviation being most pronounced in the higher concentrations. Figs. 4

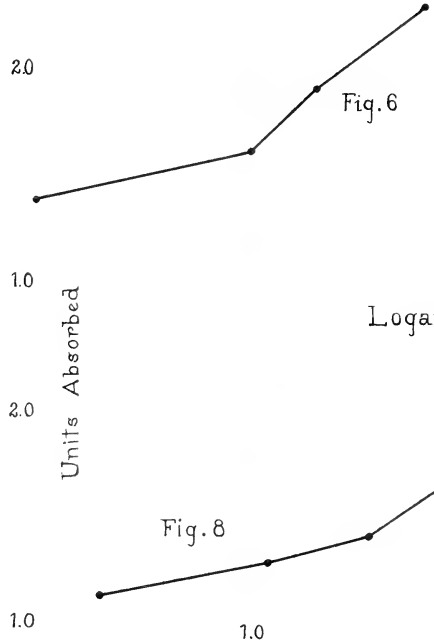
Explanation of the Figures:

Fig. 6. Logarithmic plot of the data of test 14, Table IX. $K = 24.0$, $n = 0.58$.

Fig. 7. Similar plot of test 16, Table IX. $K = 6.53$, $n = 0.56$.

Fig. 8. Similar plot of test 8, Table IX. $K = 8.0$, $n = 0.31$.

Fig. 9. Similar plot of test 7, Table IX. $K = 15.0$, $n = 0.41$.


2.0
1.0
Units Absorbed
Fig. 6
Fig. 7
Logarithmic plots
2.0
Fig. 8
Fig. 9
1.0
1.0
2.0
1.0
2.0

and 5 represent comparatively high values for K, but in neither is it so high as in curve A, Fig. 3. These plots, also, deviate from the straight line in the higher concentrations of antibody units. Figs. 6 to 9 all represent very low values for K, and in these is seen a drop in the curve in the intermediate concentrations.

This deviation of the curve from the straight line, according to a physical interpretation, means that there is a selective absorption of the antibodies, the amount of selection differing with the concentration. Selective absorption may, although does not necessarily, denote the presence of two or more substances in the serum capable of being absorbed.

It was tentatively assumed, therefore, ~~that~~ that the variations in the absorption are caused by the presence of some inhibiting substance in the serum; and that this substance exerts its influence by virtue of its own absorption by the cell. The following "multiple absorption" experiments were carried out to support or disprove this hypothesis.

Sheep cells, one unit, were allowed to take up a certain number of units of a sensitizer from a serum that, from previous tests, had been found to have a comparatively high absorption constant. (The value of

K was not determined exactly in these experiments, but only estimated to be high or low in proportion to the amount of absorption from the concentrations tested.) Another similar lot of cells was allowed to take up sensitizer from a serum having a lower absorption constant. Both lots of cells were then separated from the supernatant liquid by centrifuging at low speed, and each treated with an equal number of units of sensitizer from one serum. The amount of absorption was determined as in the preceding experiments.

If this inhibiting element is really taken up by the cells, those which had received a certain number of units of sensitizer from a serum with a low absorption constant should be more resistant to further sensitization than cells which had received an equal number of units from a serum with a higher absorption constant. The results of the experiments described below agree with the expectations.

Experiment 1:

(a). First Absorption.- Set (1) *, One unit of sheep cells was treated with 50 units of fresh guinea-pig immune serum, titer 1/150. Titration of the super-

* All sets were made in duplicate.

natant liquid showed only 5 units absorbed. The value of K, therefore, is very low. Set (2), One unit of sheep cells, treated with 50 units of guinea-pig immune serum, titer 1/5000, absorbed 20 units of sensitizer. The value for K is, therefore, much higher than for the serum of Set (1), although still rather low.

(b). Second Absorption.- Each lot of cells was then treated with 50 units of the second serum, titer 1/5000. By titration of the supernatant liquid, lot 1 was found to have taken up 5 more units, while lot 2 absorbed 10 more. The serum with the lower absorption constant was, therefore, more effective in preventing further sensitization of the cells than the serum with the higher absorption constant.

Experiment 2:

(a). First Absorption.- Set (1), One unit of cells, treated with 100 units sensitizer from rabbit serum, titer 1/1200, absorbed 50 units. The value for K is, therefore, comparatively low. Set (2), One unit of cells, treated with 100 units sensitizer from rabbit serum, titer 1/5000, took up 90 units. The value for K then is comparatively high.

(b). Second Absorption.- Each lot of cells, after separation from the supernatant liquid, was

treated with 100 units sensitizer from the second serum, titer 1/5000. Lot 1 took up so small an amount in this second absorption that it was not titratable, while lot 2 took up 25 more units. Here, 50 units taken up from the serum with the lower absorption constant were more effective in preventing further sensitization of the cells than 90 units from the second serum.

Experiment 3:

(a). First Absorption.- Set (1), One unit cells, treated with 100 units sensitizer from rabbit serum, titer 1/600, absorbed completely 100 units. The value of K is high then, in spite of the low titer. Set (2), One unit of cells, treated with 200 units sensitizer from rabbit serum, titer 1/4000, absorbed 100 units. The value for K, therefore, is much lower than for the serum of Set (1).

(b). Second Absorption.- Each lot of cells was then treated with 200 units of sensitizer from the second serum, titer 1/4000. Cells of lot 1 absorbed 75 more units, and those of lot 2 absorbed 30 more. The serum with the lower absorption constant is here, also, the more effective in making the cells resistant to further sensitization.

Experiment 4:

(a). First Absorption.- Set (1), One unit of

cells, treated with 200 units of sensitizer from rabbit serum, titer 1/4000, absorbed 80 units. The absorption constant is very low. Set (2), One unit of cells, treated with 120 units sensitizer from rabbit serum, titer 1/500 , took up 120. The absorption constant, therefore, must be very high.

(b). Second Absorption.- Each lot of cells, after separation from the supernatant liquid, were treated with 120 units from the second rabbit serum, titer 1/500. Lot 1 absorbed 40 units more, while lot 2 took up 110 more. In this case, also, the absorption from the serum having the lower value for K is much more effective in making the cells resistant to further sensitization than absorption from the serum with the high value for K.

In every case, in the foregoing experiments, the antibodies taken up by the cells from the serum with the lower absorption constant had much more influence in inhibiting further sensitization of the cells than a larger number of antibodies taken up from the serum with the higher absorption constant. It is obvious, therefore, that the inhibiting element, whatever its nature, can be taken up by the cells, and and probably in proportion to its combining affinity, inhibits the absorption of the antibodies.

VIII. Variation of the Absorption with the Length of the Period of Immunity.

In the foregoing section, it was found that the disturbing element, which inhibits the absorption of the antibodies, is actually taken up by the cells along with the antibodies. In Section VI, it was indicated that the amount of this substance probably varied during the time that the animal is immune. In this Section, it is purposed to show how the absorption varies during the immunity of the animal, which variation seems to give some indication as to the nature of the disturbing factor.

A careful record of rabbits 481 and 486 was kept while testing for the relation of the serum proteins to absorption. The animals were given three injections within the first nine days and no more during the 116 days they were under observation. Table XI shows the fluctuations in the absorption from their sera, also the titers and the number of days after the first injection when the serum was drawn. In Figs. 10 and 11 are plotted the observations.

The first tests, made on the 16th day, showed a high titer and a comparatively high absorption. The second test, on the 24th day, showed a great drop in

Table XI. Absorption Tests on the Sera of Rabbits 481 and 486, Drawn at Intervals During a Period of Immunity of 116 Days. *

Concentration of antibody units per cc. in the serum dilutions.	50	100	200	500	1000	Titer	Days immune
Units absorbed from serum of							
Rabbit 481,							
Test # 1		94	175	400	600	10000	16
" # 2	30	50	91	160		1500	24
" # 3	30	55	100			800	62
" # 4	23	37	81			600	80
" # 5	30	45	98			500	91
" # 6	30	50	110			500	110
" # 7	42	76	140	330		500	116
Rabbit 486,							
Test # 1		94	180	410	670	8000	16
" # 2	30	52	91	160		1500	24
" # 3	40	80	150			1000	62
" # 4	38	71				800	80
" # 5	30	55	102			500	91
" # 6	35	65	120			600	110
" # 7	40	71	135	370		600	116

* These rabbits received three injections of washed sheep corpuscles within the first nine days. Each injection consisted of 3 cc. of the cells, measured in terms of whole blood.

Table XII. Absorption Tests on the Sera of Rabbit 69 and a Goat, Drawn at Intervals During the Period of Immunity.

Concentration of antibody units per cc. in the serum dilutions.	50	100	200	500	1000	Titer	Days immune.
Units absorbed from serum of							
Rabbit 69, *							
Test # 1		99	194	450		1000	6
" # 2			198	485	875	20000	10
" # 3			194	452	690	16000	16
" # 4		60	100	200	300	2000	27
" # 5		75	120	250	400	1000	37
" # 6		75	125	250		800	45
" # 7		75	130	275	400	600	62
" # 8		96	150	350	500	600	96
Goat, **							
Test # 1	49	96	180			1000	10
" # 2	20	30	70			2000	19
" # 3	40	70	100			1000	49
" # 4	40	70				1000	63
" # 5	40	70	100			1000	65
" # 6	45	75	120			1000	92
" # 7	47	85	150			800	120

* Rabbit 69 received two injections with a six-day interval.

** The goat received three large injections of about 8 cc. each and ten very small injections of about 1.5 cc. each. The last injection was one month before the final test.

Explanation of the Figures:

Fig. 10. Plots of the absorption from the serum of rabbit 481 against the time of immunity. See Table XI. Curve A represents the absorption from 200 units, B, from 100 units, and Curve C represents the square root of the titer.

Fig. 11. Similar plot for rabbit 486, Table XI. Curve C represents the absorption from 50 units, and D represents the square root of the titer.

Fig. 12. Similar plot for rabbit 69, Table XII. Curve A represents the absorption from 500 units, and B from 200 units. The titers of the sera on the various days is indicated at the top of the chart.

Fig. 13. Plot similar to Fig. 11, for the goat. See Table XII.

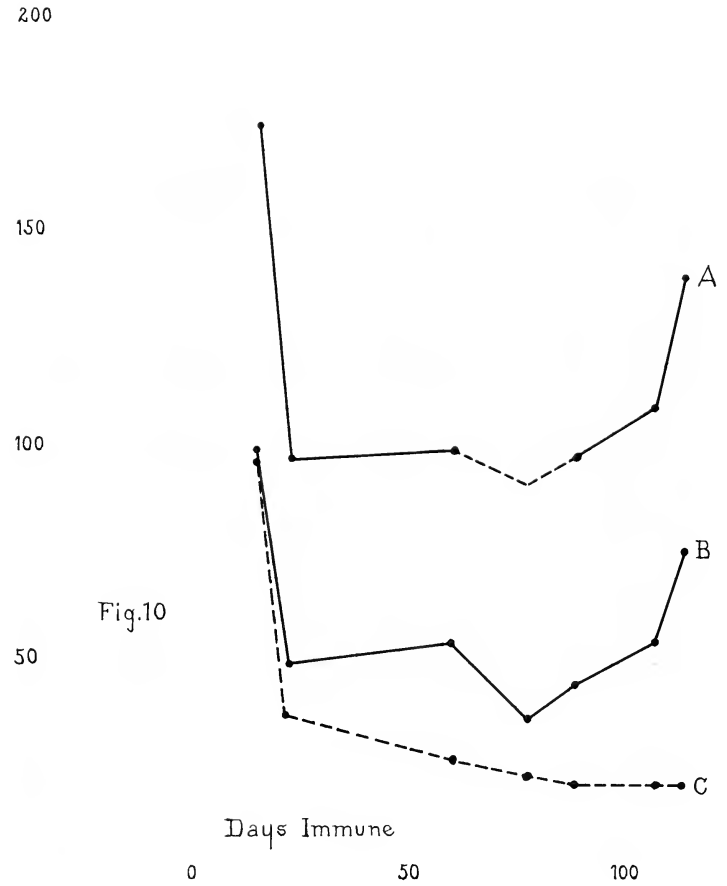


Fig.10

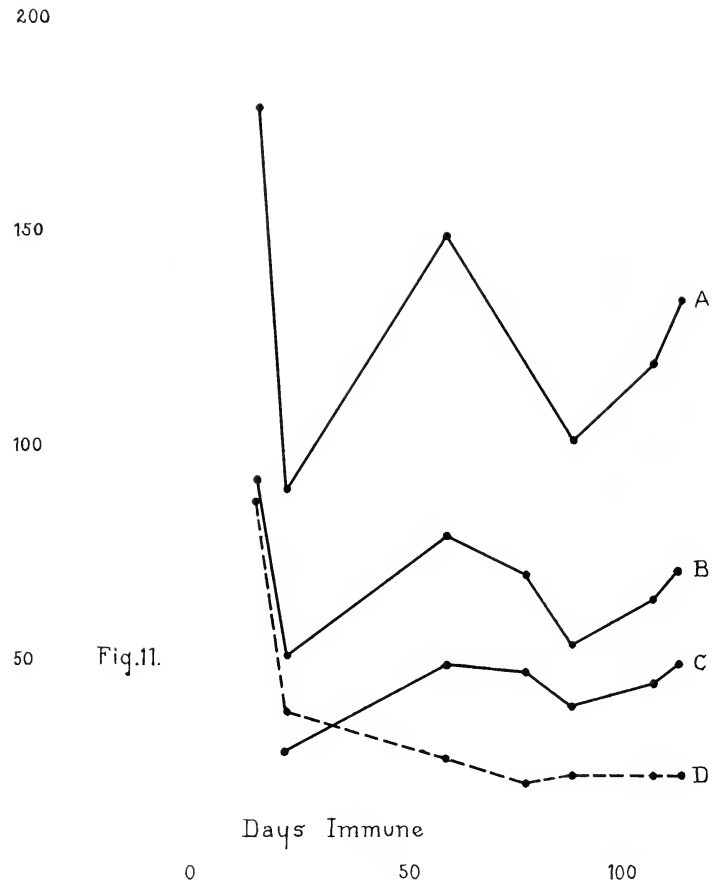


Fig.11.

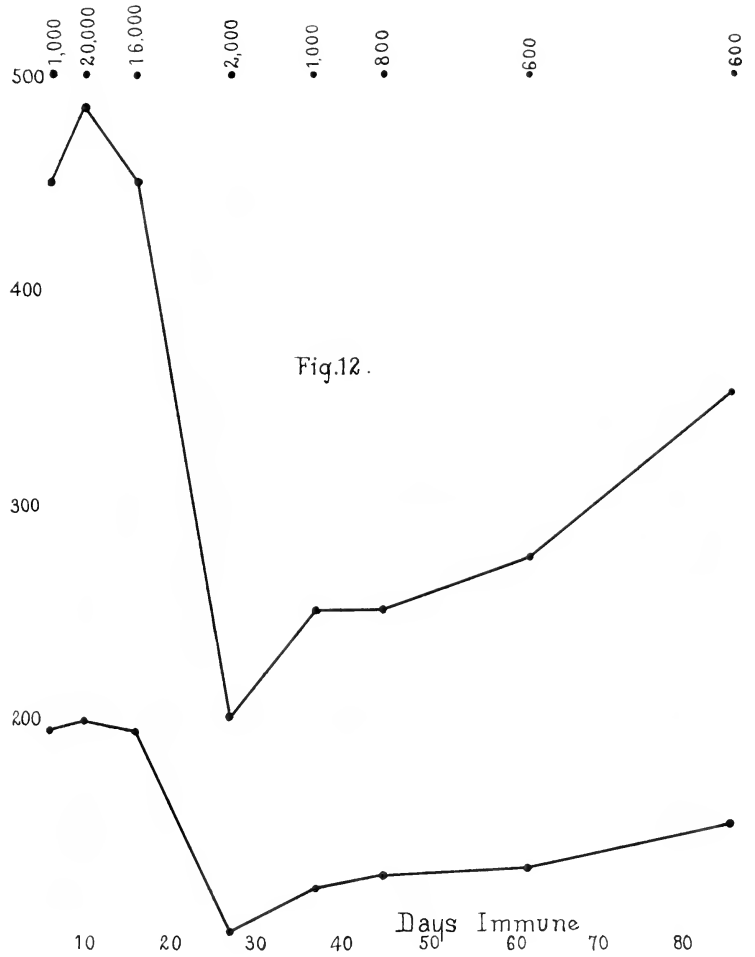

1,000
20,000
16,000
2,000
1,000
800
600
600
500
400
300
200
10
20
30
40
50
60
70
80
Days Immune

Fig.12.

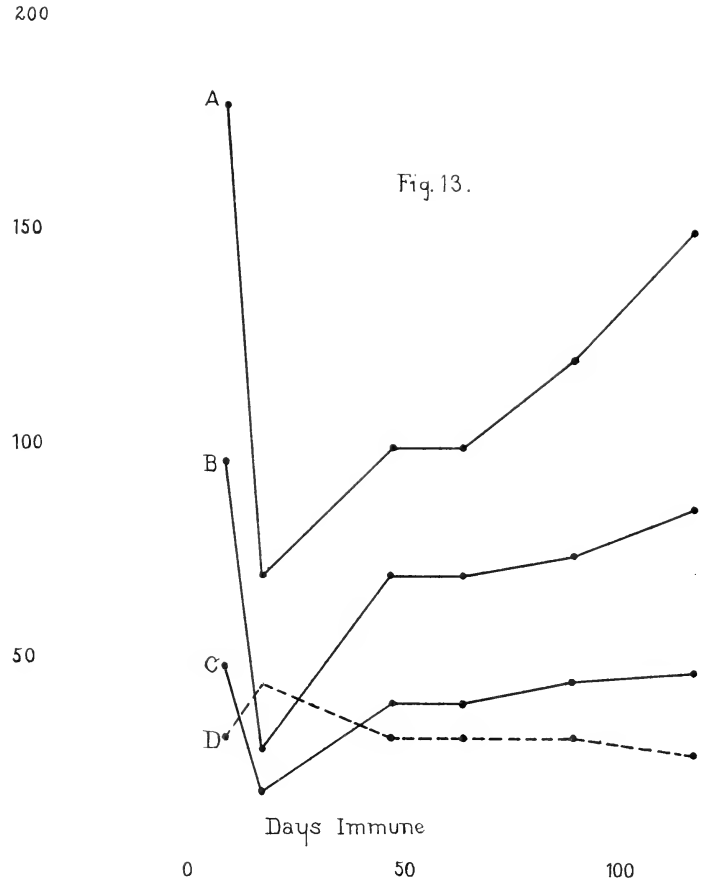


Fig. 13.

the titer and a corresponding drop in the absorption. Following the initial drop, the titer dropped very gradually until about the 32nd day, and from then on held fairly constant until the last observation on the 116th day. Further observations were not possible on account of the death of the animals. In the case of rabbit 481, where the titer dropped gradually, or held almost constant, the absorption, although fluctuating somewhat, also held fairly constant, and toward the end of the period of observation, showed a distinct rise. The fluctuations of the absorption from the serum of rabbit 486 were more pronounced, showing a marked rise after the initial drop, with a rise at the end. It is highly probable, however, judging from other observations described below, that the rise at the end would have continued higher, had it been possible to make further tests.

The results from these two rabbits seemed significant, so a number of others were started. Unfortunately, all except one, rabbit 69, died so their history is not recorded here. A goat was also immunized and its history followed. Table XII and Figs. 12 and 13 show the observations on these animals.

The results agree very well with those obtained

with the other two rabbits. Even though the titer is not high at first, the absorption is high, with a great drop within the second or third week of the period of immunity. Following this, the absorption holds about constant for a time, after which it shows a more or less gradual rise.

The history of these animals seems highly significant in suggesting an explanation as to the nature of the disturbing factor in the absorption. In nearly every case, the sudden initial drop in the absorption is accompanied by a similar drop in titer. In the case of the goat, this is not evident, but nine days had elapsed between the first and second tests, during which the titer may have gone higher and dropped back to 1/2000.

It is conceivable that,as the amboceptor content of the animal's blood-stream drops, the destruction of the antibody occurs in progressive stages, the first stage being merely the loss of the power to sensitize the cells to the action of complement. The ability to combine with the cell would still be retained for a time. These would be, in effect, somewhat analogous to the "agglutinoids" of Ehrlich, which were worked out so thoroughly by Eisenberg and Volk (22). In titrating the serum, then, these deterioration products of the

amboceptor would not be detected, and one hemolyzing unit would contain, in addition to the antibodies, a variable amount of the deterioration products. Those which still retained their affinity for the antigen would, in the absorption experiments, tend to cause a lowering of the absorption of the antibodies. The fact that the greatest lowering of the absorption is coincident with the most rapid destruction of the antibodies is very forcibly suggestive that the substance in the serum which causes this disturbance may be defined as the products of the destruction of the antibodies.

Discussion.

It has been found that, in the absorption of hemolytic immune bodies by red cells, immense quantities, as much as 2000 units or more, may be taken up by one unit of cells, provided their concentration in the surrounding medium is sufficiently high. On the other hand, all the antibodies are often not taken up even from concentrations as low as 50 units per cc. This is certainly not in accordance with the theory of chemical valencies. It has been found, moreover, that this absorption follows, more or less strictly, the physical law proposed by Arrhenius, which applies to the distribution of a solute between two immiscible solvents. The equation, $B = K C^n$, which represents this law, could, however, with equal accuracy, be applied to simple adsorption phenomena, such as the taking up of acetic acid from a benzol solution by silica gel. Arrhenius assumed that all absorption tests with the hemolytic system would follow this law within the limits of experimental error, but it was shown by Manwaring, and confirmed in this paper, that many, in fact, most of the absorption tests do not follow this law strictly, as is shown by the deviation of the logarithmic curves

from the straight line.

One of the most striking things about the absorption of hemolytic antibodies is the great differences obtained with the different sera. While with many sera the absorption is very low, incomplete even from a concentration of antibodies as low as 20 units per cc., with other sera the absorption is practically complete from as much as 200 units per cc.

The antigen-antibody combination takes place with great rapidity. A large proportion of the antibodies are absorbed almost instantly. Equilibrium is almost established within five minutes, and in every case within fifteen minutes with any concentration of the antibodies.

The variability of the absorption from the different sera is shown also in the extraction at the various temperatures. The extraction is always less at 0° than at room temperature, and becomes progressively less again as the temperature is raised to 45° and above. The differences in the different sera become evident in the absorption at room temperature and at 37°. Of the sera that were tested, the majority gave the highest extraction at 37° - 40°. Others showed the highest extraction at about room temperature, 15° - 25°. This

indicates that the optimum temperature for the antigen-antibody combination is a variable, the absolute value of which depends on the particular lot of serum used in the test.

An endeavor was made to determine the cause of the extreme variations obtained in the absorption from the different lots of serum. A large number of tests were made on the different sera, at the same time making quantitative determinations of the globulins and albumins. The quantitative fluctuations of the serum proteins were not shown to bear any direct relation to the variations in the absorption. They may, however, exert some more indirect influence which was not detected by this method.

Although in many cases, the serum with the lower titer gave the lower absorption, this was not constant, and with titers of 2,000 units per cc. or higher the differences did not seem to bear any relation to the amount of sensitizer taken up by the cells. Normal sera and sera that were drawn early in the period of immunity gave uniformly high absorption, regardless of the titer. This indicates that the lower absorption comes about as a result of some change in the serum after the animal is immunized.

This lowered absorption is evidently due to

some substance in the serum which is absorbed by the cells along with the sensitizer; it is probable, also, that, in proportion to the combining affinity of this substance, the absorption of the antibodies is inhibited. This is shown by the fact that cells, which take up sensitizer from a serum that gives a low absorption, are more resistant to further sensitization than an equivalent lot of cells sensitized with a larger number of antibody units from a serum that gives a high absorption. The serum, therefore, that gives the higher absorption, has the smaller percentage of this inhibiting substance, as compared to the number of antibodies.

It is true that the number of animals is not large, for which the history was kept of the absorption at intervals during the period of immunity. The results, however, are consistent. The fact that, in every case, the absorption is highest early in the immunity period, and that the greatest drop in the absorption is coincident with the most rapid drop in titer, is forcibly suggestive that the inhibiting substance represents the deterioration products of the amboceptor, i.e., the amboceptors which have lost their sensitizing power, but still retain their combining affinity for the antigen. This would be, in effect, somewhat analogous to the

"agglutinoids" of Ehrlich. This hypothesis would account, not only, for the great differences in the absorption from the different sera, but also for the fact that so many of the tests fail to conform to the equation proposed by Arrhenius.

Summary.

1. Red cells, when treated with their specific immune serum, take up amounts of sensitizer which vary with the concentration of the sensitizer in the surrounding medium. Although from very low concentrations, all is not absorbed, as much as 2,000 units or more may be taken up by one unit of cells, provided the concentration of the antibodies is sufficiently high.

The logarithmic plots of some of the tests approach a straight line very closely, while many deviate considerably from the straight line. The amount of deviation of the curve is proportional to the amount of deviation of the tests from the physical law proposed by Arrhenius, which is expressed by the equation, $B = K C^n$. The amount of absorption varies also with the serum used. Some give practically complete extraction from as much as 200 units per cc., while with other sera, all is not absorbed from concentrations of antibodies as low as 50 units per cc.

2. The combination of the red cell and its antibody is extremely rapid. In every case, fifteen minutes were found to be sufficient time for equilibrium to be established.

3. With the majority of sera the extraction of sensitizer by the cells was found to be greatest at a temperature of about 37°. Some sera, however, gave more complete absorption at 15° to 25° than at any other temperature.

4. Neither the quantitative fluctuations of the serum proteins nor the titer of the serum was found to have any constant relation to the variations in the absorption.

5. Cells, sensitized with a serum which gave a low absorption, were more resistant to further sensitization than cells, sensitized with a much larger number of units from a serum which gave a high absorption. This indicates that the substance in the serum, that inhibits the absorption, is really absorbed by the cells along with the antibodies.

6. In every case tested, the extraction of the antibodies was greatest from serum drawn early in the period of immunity, regardless of the titer. The greatest drop in the absorption was found to be coincident with the most rapid drop in the titer.

7. The accumulated evidence is forcibly suggestive that the great variations in absorption are

due to the presence of the deterioration products of the antibodies, i.e., the antibodies which have lost their sensitizing power but still retain their affinity for the antigen.

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In October 1913 he entered the Indiana State Normal School, and, after four years of study, received the A.B. degree. For two years of this time he was student assistant in the Department of Zoology, where his major work was done.

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